Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Detection of Mycoplasma Contamination

Date: April 21, 1999

Supersedes: New

Number: STSAM0910.01

Standard Requirement: 9 CFR, Part 113.28

Contact Person: Gerald Christianson (515) 663-7416

Approvals:

_____ Date:_____
Gerald G. Christianson, Head
Cytology and Sterility Section

_____ Date:_____
P. Frank Ross, Acting Quality Assurance Manager

_____ Date:_____
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Supplemental Assay Method for Detection of Mycoplasma Contamination

Table of Contents

Supplemental Assay Method for Detection of Mycoplasma Contamination

1. Introduction

This Supplemental Assay Method (SAM) describes the test procedure used to detect Mycoplasma contamination in live viral products, master cell stocks, and Master Seed Viruses, as prescribed in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.28 (028-PU0). If Mycoplasma contamination is present, colonies will form on the agar as seen under a stereoscope.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Biosafety cabinet

2.1.2 Dissecting stereoscope (200 magnification)

2.1.3 30°-35°C incubator (humidified 4-6% CO_2)

2.1.4 Pipet-aid or equivalent

2.1.5 Bunsen burner

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Mycoplasma broth (**Section 8.1**)

2.2.2 Mycoplasma agar in petri dishes, 15 x 60 mm (**Section 8.2**)

2.2.3 DPN/L cysteine (**Section 8.3**)

2.2.4 70% ethyl alcohol

2.2.5 0.05% Germ Warfare disinfectant

2.2.6 Glassware: tubes, flasks, and petri dishes

2.2.7 Sterile clothes: coveralls (or frock, or lab coat), mask, hair bonnet, sleeves, gloves, and protective eye wear

2.2.8 Sterile gauze pads, 4 x 4 in

2.2.9 Clean-Pal wipes

2.2.10 Individually packaged sterile syringes, 1 cc, 3 cc, 5 cc, and 10 cc

2.2.11 Pipettes, 1 cc

3. Preparation for the test

3.1 Personnel qualifications/training

The personnel performing the test must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. The personnel must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

3.2.1 Turn the biosafety cabinets on 1 hr prior to the test session.

3.2.2 Monitor the incubators daily for temperature according to the current version of NVSLSOP0002.

3.2.3 Monitor freezers and coolers used for the storage of biologicals for temperature daily, according to the current version of GDOCSOP0002.

3.3 Preparation of reagents/control procedures

3.3.1 Determine the growth-promoting qualities of the Mycoplasma broth and agar as required in 9 CFR 113.28(d)(4). Use *Mycoplasma hyorhinis* and *Acholeplasma laidlawii* as the positive controls for this test procedure. Conduct these positive control tests on each lot of media according to the current version of STPRO0010.

3.3.2 Prepare the *M. hyorhinis* and *A. laidlawii* reagents by the current version of the STRPP0005 protocol.

3.3.3 Positive Controls: Inoculate 10 vessels containing 9 ml Mycoplasma broth with 1 ml each of an end point dilution of *A. laidlawii* and 10 more test vessels with the next lowest tenfold dilution of *A. laidlawii,* as described in the current version of STPRO0010. Read the test vessels as positive for growth if a dark precipitate is seen on the bottom of the vessel. Record this growth (# positive/10 at each dilution) on day 14 of the test. Dilute a vial of *M. hyorhinis* culture to its end point. Inoculate 1 flask of 100 ml of Mycoplasma broth (to which 2 ml of DPN/L cysteine has been added) with 1 ml of the end point dilution as determined by using the current version of STPRO0010. Inoculate 2 agar plates with 0.1 ml of the end point dilution and 2 plates with 0.1 ml of the next lowest dilution. Tilt the plates to allow the inoculum to flow over the surface. Read these plates after 10 days of incubation. Subculture the positive control flask in the same way as each subculture is done from a product's flask (**Section 4.11**).

3.3.4 Negative or Media Controls: Incubate 1 representative vessel of uninoculated broth to confirm the sterility of the media (9 CFR 113.28(c)(4)). Inoculate 1 plate per subculture (**Section 4.11**) from this uninoculated vessel to confirm the broth and agar purity from Mycoplasma contamination.

3.4 Preparation of the samples

3.4.1 Receive the biological samples to be tested from the Biological Materials Processing Section (BMPS) according to the current version of STSOP0001.

3.4.2 Log in the biological samples by assigning a test number and completing the testing log book as stated in the current version of STSOP0011.

3.4.3 Order sufficient Mycoplasma broth from the media preparation department as is needed for the test and the rehydration of the product according to the outline. This broth should arrive each Tuesday as a standing order of flasks and tubes. The standing order

provides sufficient media for 12 serials, as well as positive and negative controls. Order the Mycoplasma agar each week in sufficient quantities for new serials and consequent subcultures to be inoculated the next week.

4. Performance of the test

4.1 On the day of the test, disinfect the vials of biologics with 0.05% Germ Warfare, using a Clean-Pal. Clean the tops of the vials and rubber stoppers especially well.

4.2 Place these disinfected vials on a tray and place the tray under the biosafety hood in the Mycoplasma testing room.

4.3 Gown up for doing the Mycoplasma test by wearing sterile coveralls or frock, sleeves, mask, bonnet, gloves, and protective eye wear.

4.4 Wipe down the interior surfaces of the biosafety cabinet used for testing with 70% alcohol.

4.5 Number the media test vessels and agar plates to coincide with the serials to be tested.

4.6 Place the testing materials (syringes, pipettes, 4 x 4-in gauze squares) in the biosafety cabinet or on a cart next to the cabinet.

4.7 Add 2 ml of DPN/L cysteine to each flask of broth to be used for testing.

4.8 Place the test media for the serials to be tested in the biosafety cabinet.

4.9 Disinfect the tops of the samples with a 4 x 4-in gauze pad soaked in 70% alcohol. Flame the tops of the samples using a Bunsen burner.

4.10 One by one, rehydrate each serial, if desiccated, using a syringe and needle. Rehydrate all products with Mycoplasma broth as the diluent. Rehydrate products used for mass inoculation by water or spray with 30 ml of Mycoplasma broth per 1000 doses.

4.11 Inoculation of plate. Place 0.1 ml of product on an agar plate and make a short continuous "Z" streak across the plate with a pipette. Tilt the plate in several directions to allow the inoculum to flow over the surface. Leave the plate in the closed, upright position under the hood for approximately 30 minutes until the inoculum is dry. Invert, cover, and place the plates in a humidified, 4-6% CO_2 incubator at 33^0-37^0C for 10-14 days.

4.12 Inoculate a flask containing 100 ml of Mycoplasma broth, to which 2 ml DPN/L cysteine has been added, with 1 ml of product and mix thoroughly. Incubate the flask at 33^0-37^0C for 14 days. During this time, streak 4 agar plates with 0.1 ml each of broth from the incubated flask of inoculated medium on the following days: 1 on the 3rd day, 1 on the 7th day, 1 on the 10th day, and 1 on the 14th day postinoculation.

4.13 Repeat the procedures in **Sections 4.7-4.12** with the other serials of biologic to be tested this day. Initial and date the testing log book after completing the testing.

4.14 Incubate the agar plates for 10-14 days and then examine them with a stereoscopic microscope at 25X.

4.15 With Master Seed, cell, or service samples, inoculate additional agar plates according to the current version of STPRO0022 and conduct the VERO cell procedure according to the current version of STPRO0005 on these samples as well.

4.16 Clean up the Mycoplasma room by wiping down the interior of the biosafety cabinet and counter tops with 70% alcohol. Discard the biological samples and any extra media by autoclaving.

5. Interpretation of the test results

5.1 At 10 days postinoculation (13 days after the 4th subculture), examine the plates for Mycoplasma colonies using a stereoscope. If Mycoplasma colony growth appears on the positive control plates and does not appear on the negative control plates, the test is valid. If any Mycoplasma colonies are found on the initial day agar plate or any of the subculture agar plates, the serial is positive for Mycoplasma contamination and is unsatisfactory (UNS). Positive contaminants may be further tested for tentative

identification according to the current versions of STPRO0006, STPRO0007, and STPRO0015.

5.2 If, upon examination of all the plates, no Mycoplasma colonies are found, the serial is determined to be negative for Mycoplasma contamination and is satisfactory (SAT).

5.3 If, at any time during the test, mold or bacteria overgrows any plate, the Mycoplasma test for that serial must be considered a no-test (NT), since Mycoplasma contamination could not be determined.

6. Report of test results

6.1 Record the test conclusions of SAT, UNS, or NT (based on the corresponding test results of positive, negative, or inconclusive Mycoplasma contamination) in the testing log book and on the computer test sheet for each serial tested. Initial and date the log book and computer test sheet after entering the test results on the 28th day.

6.2 Enter the results and conclusions in the computer, according to the current version of STSOP0021. A computer printout of the result and conclusion for each serial tested will be generated. Compare these printouts against the test sheet and log book for accuracy.

6.3 Forward the test report printouts, log book, and computer test sheet to the CY/ST supervisor or microbiologist to check, sign, and date.

6.4 Validate each test report in the computer according to the current version of STSOP0021.

6.5 File the signed and validated test report printouts in the sterility file cabinet under the first 2 numbers of each serial's product code. File the BMPS test sheet by test code in the same file drawer.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.28, U.S. Government Printing Office, Washington, DC, 1998.

8. Appendices

8.1 Media Formulation NVSL # 10162

MYCOPLASMA (MG) BROTH

Heart Infusion Broth	25	g	(DIFCO)
Proteose Peptone #3	10	g	(DIFCO)
Yeast Extract	5	ml	(GIBCO SOLUTION)
1% Thallium Acetate	25	ml	(FISHER)
1% Tetrazolium Chloride	5.5	ml	(FISHER)
Penicillin (100,000 u/cc)	5	ml	(USB)
Horse Serum (Heat Inactivate.)	100	ml	(SIGMA)
QH_2O	970	ml	

Adjust pH to 7.9 with 10% NaOH
Filter through O.2-μm sterilized mini capsule filter (Gelman Sciences).
Media must be prepared in a sterile room.

8.2 Media Formulation NVSL # 10167

MYCOPLASMA (MG) AGAR

Heart Infusion Agar	25	g	(Difco)
Heart Infusion Broth	10	g	(Difco)
Proteose Peptone #3	10	g	(Difco)
1% Thallium Acetate	25	ml	(Fisher)
QH_2O	995	ml	

Heat to boiling.
Cool and adjust pH to 7.9 with 10% NaOH.
Autoclave 20 min.
Cool to 56°C and add:

Horse Serum (HI)	126 ml	(Sigma)
Yeast Extract (Sterile)	5 ml	(Gibco solution)
0.5% Penicillin	5.2 ml	(USB)
1% DPN-Cysteine	21 ml	
	TOTAL	(1152 ml)

Media must be prepared in a sterile room.

8.3 Media Formulation NVSL # 30039

DPN/L-CYSTEINE

Supplemental Assay Method for Detection of Mycoplasma Contamination

Nicotinamide-adenine-dinucleotide (DPN, NAD)	5 g(ICN)
Q.S. H_2O to	500 ml
L-Cysteine	5 g(Calbiochem)
Q.S. H_2O to	500 ml

Mix each chemical separately until dissolved.
Pour solutions together and let mix.
Filter and dispense.